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Mycoplasma hominis and *Trichomonas vaginalis*: a unique case of symbiotic relationship between two obligate human parasites

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1. ABSTRACT

Mollicutes are the smallest and simplest self-replicating microorganisms. Despite the minimal genome and apparent lack of complexity, mycoplasmas show a high degree of adaptation to the most diverse environments. *Mycoplasma hominis* is a human sexually transmitted mycoplasma which is able to establish a biological association with *Trichomonas vaginalis*, a pathogenic flagellated protist. *M.hominis* and *T.vaginalis* share the same specific natural niche, the human genitourinary tract. Symbiotic relationships between unicellular eukaryotes and bacteria are well known and have been extensively studied, providing interesting insights into the biology of one or both the symbionts. The relationship between *T.vaginalis* and *M.hominis* is unique in that it was the first described association of two obligated human parasites. Several aspects of this relationship have been investigated, showing how the trichomonad may be viewed not only as a new niche for *M.hominis*, but also as a "Trojan horse" for the transmission of the bacterial infection to the human host.

2. INTRODUCTION

2.1. The relationships between protozoa and bacteria

Symbiotic relationships between environmental protists and pathogenic bacteria are well known and quite common in nature (1). Nevertheless the role of symbiosis between protists and prokaryotes in human infection has received little attention until the finding that the symbiosis itself may influence the pathogenicity of one or both the microorganisms involved (2). Unicellular eukaryotes are frequently isolated from the most diverse ecosystems, where they predate bacteria in environmental biofilms. Various bacteria have developed diverse mechanisms to survive protist killing. Also several human bacterial pathogens (e.g. *Legionella pneumophila*, *Mycobacterium* spp., *Francisella tularensis, Salmonella* spp. and *Vibrio cholerae*) have been shown to infect and replicate within protists (3, 4). The relationship between *L. pneumophila*, the causative agent of Legionnaire's disease, and environmental amoebae is the most intensively studied (5). *L. pneumophila* is able to survive and to multiply within

environmental amoebae and shows an enhanced infectivity and virulence when released from the protozoan host (6, 7, 8). A growing body of evidence shows the influence of protozoan hosts over the virulence of the bacterial symbionts. Indeed protists appear to play a key role in the passage of pathogenic bacteria from the environment to their human host, acting as vectors for bacterial infection. According to several authors, protists may be viewed as "biological gyms" for pathogenic bacteria, where they train to avoid eukaryotic killing mechanisms (9, 1, 3).

The existence of a symbiotic relationship between *Trichomonas vaginalis* and *Mycoplasma hominis*, which is the first reported example of symbiosis between two obligate human pathogens, has been recently reported by our research group

2.2. *Mycoplasma hominis* and *Trichomonas vaginalis*: two sexually transmitted human pathogens

Trichomonas vaginalis is a flagellated parasitic protist responsible for trichomoniasis, one of the most common sexually transmitted diseases in humans estimated to affect at least 200 million people worldwide (10). *T.vaginalis* is responsible for severe vaginitis accompanied by abdominal pain, itching, and foul-smelling discharge (11). *T.vaginalis* infection is mainly asymptomatic in men (12). Moreover, trichomoniasis is associated with an enhanced risk of neoplastic transformation in cervical tissues (13) and increased human immunodeficiency virus (HIV) seroconversion in women (14, 15). The mechanisms by which *T.vaginalis* exerts its pathogenic effects involve adhesion to host cells (16, 17, 18), the activity of pH-dependent pore-forming proteins (19, 20) and of cytoskeleton-disrupting proteases (21).

Genital infection by *M.hominis* is associated with a variety of signs and symptoms, but the pathogenicity mechanisms of this bacterium are still unclear. *M.hominis* can be isolated from the genital tract of both symptomatic and asymptomatic individuals and it is considered a commensal microoorganism of the genital tract that can act as a pathogen. A major diagnostic problem is therefore to define its effective role as a cause of infection. Nevertheless, there are evidences that *M.hominis* may play an important etiologic role not only in genital tract diseases of both men and women, but also in extragenital infections (22, 23). Interestingly, both *M.hominis* infections and trichomoniasis are associated with several pregnancy and post-partum complications, including pre-term delivery and low birth weight infants (24, 25, 26, 27).

3. THE BIOLOGICAL ASSOCIATION BETWEEN *MYCOPLASMA HOMINIS* AND *TRICHOMONAS VAGINALIS*

A number of morphological, biological and clinical observations induced our research group to investigate on the existence of a biological association between *T.vaginalis* and mycoplasmas:

I. Electron microscopy studies showed the presence of apparently intact mycoplasmas in food vacuoles of freshly isolated *T.vaginalis* cells, even after 6 weeks of cultivation (28, 29).

II. *In vitro* cultures of the protist *Plasmodium falciparum* may be accidentally infected by mycoplasma species commonly found as cell culture contaminants (30).

III. Large epidemiological studies on the prevalence of different sexually transmitted diseases highlighted a strong clinical association between *T.vaginalis* and *M.hominis* infections. Studies carried out on more than 40.000 individuals showed that an unexpected large percentage of patients affected by trichomoniasis were also positive for *M.hominis* (31, 32). The association is strictly species-specific, since is not observed with *Ureaplasma urealyticum*, an other Mollicutes species that is a much more common inhabitant of the human genital tract.

We investigated on a group of more than 200 symptomatic women for the association among *T.vaginalis, U.urealyticum* and *M.hominis,* using both traditional and molecular techniques. Results confirmed the exclusive association between *T.vaginalis* and *M.hominis* (unpublished data).

PCR analysis, standard biochemical techniques and growth characteristics in selective media showed that more than 90% of clinical isolates from our *T.vaginalis* collection were positive for *M.hominis***.** The presence of mycoplasma species commonly found as cell culture contaminants (*M. arginini, M. hyorhinis, M. orale, M. fermentans, and Acholeplasma laidlawii*) or frequently present in the genital tract (*U. urealyticum* and *M. genitalium*) was excluded by DNA analysis. Moreover, *M.hominis* infection was still detectable after 3 months of continuous *in vitro* cultivation of protozoa in Diamond's TYM culture medium. This result shows that *M.hominis* is able not only to infect *T.vaginalis*, but also to replicate in association to protozoan cells (33). The association with *T.vaginalis* appears to be necessary for mycoplasma *in vitro* replication. Indeed, Diamond's TYM medium is not able to sustain *M.hominis* growth.

Previous studies on *T.vaginalis/M.hominis in vitro* interaction led to somehow different results (34). Taylor-Robinson and colleagues reported that, when infecting *in vitro T.vaginalis* with *M.hominis,* most bacteria were ingested and killed within 3 hours. This observation is not necessarily in contrast with our findings. In fact, upon co-incubation of a *Mycoplasma*-free *T.vaginalis* culture and *M.hominis in vitro*, we observed a rapid decrease in the number of bacteria. In a few days the number of mycoplasmas increases, reaching a *T.vaginalis/M.hominis* ratio characteristic for each strain. We detected *M.hominis* in protozoan fresh isolates, indicating that the association between the two microorganisms takes place in the urogenital tract (35). The absence of *M.hominis* in some of the *T.vaginalis* isolates analyzed may reflect a different susceptibility of protozoan isolates to bacterial infection, or

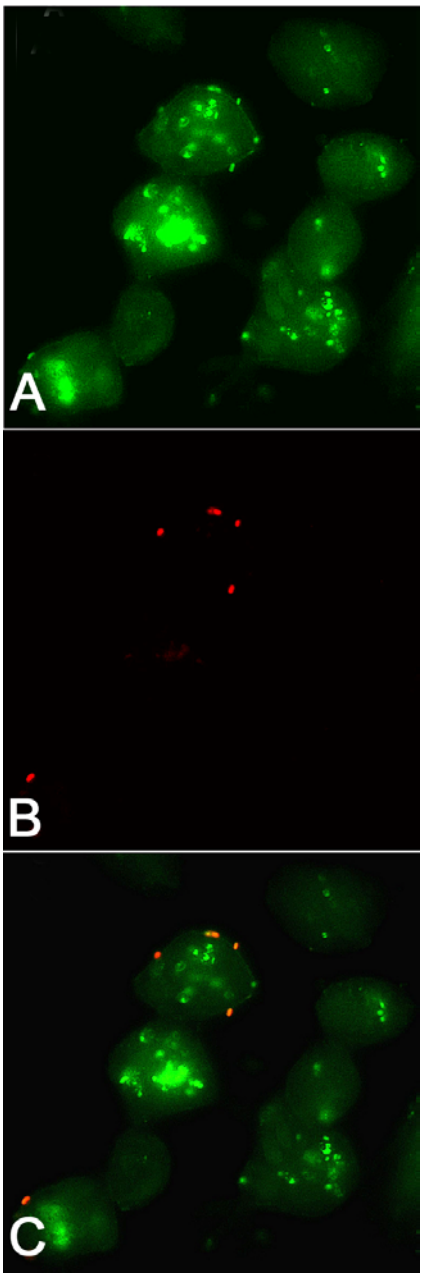

Figure 1. Micrographs depicting the cellular localization of *M.hominis* infecting *T.vaginalis*. Panels A to C represent the same area of a protozoan monolayer. Mycoplasmas were localized with anti-*M.hominis* rabbit antiserum before permeabilization of trichomonad cells, followed by staining with rodamine-conjugated anti-rabbit antibody. After permeabilization of *T.vaginalis* cells, intracellular mycoplasmas were detected with anti-*M.hominis* rabbit antiserum, followed by staining with FITC-conjugated anti-rabbit antibody. A. FITC fluorescence showing extracellular and intracellular mycoplasmas. B. Rodamine fluorescence showing mycoplasmas which are extracellularly located. C. Superimposed images of panels A and B indicating the localization of extracellular (red) and intracellular (green) mycoplasmas. Reproduced with permission of American Society for Microbiology (ASM) (44).

merely indicates that the protist never encountered *M.hominis in vivo.* This is the first case reported of a symbiotic relationship between two obligated human parasites. The presence of *M.hominis* in association with *T.vaginalis* has important clinical implications. When diagnosing a *T.vaginalis* infection, the possibility of a *M.hominis* infection should be taken into account by physicians.

The association between *T.vaginalis* and *M.hominis* differs from symbiotic relationships between environmental protists and intracellular bacteria in several distinctive features. Firstly, *T.vaginalis,* unlike environmental amoebae, is an obligated parasite unable to survive outside the human host. In fact *T.vaginalis* shows neither transformation into cystic forms nor a free-living stage. Secondly, mycoplasmas represent an atypical class of bacteria with a small genome size lacking a cell wall and several metabolic pathways. These unique characteristics are reflected in the strong dependence of mycoplasmas on host cell environment. The complete genome sequences of several Mollicutes revealed the genetic basis of such dependence. Mycoplasmas lack genes involved in aminoacid and cofactor biosynthesis. The number of genes involved in lipid metabolism and purine and pyrimidine synthesis is extremely low. Mycoplasmas are not able to synthesize fatty acids and must import nucleic acid precursors. Furthermore, mycoplasmas show a deficiency in genes coding for components of energy metabolism (36-40). Owing to this saving in gene complexity, mycoplasmas show a strong dependence on host cells and a strict host and tissue specificity (41). An intriguing issue raised by comparative genomics of mycoplasmas is that of reductive evolution. The low genomic complexity is believed to be the consequence of the intracellular parasitic lifestyle of mycoplasmas. Life in a nutrient-rich environment might have represented an evolutive pressure leading to the loss of genes (42).

In the last few years several aspects of the symbiosis between *T.vaginalis* and *M.hominis* have been characterized. *T.vaginalis* displays an isolate-to-isolate variability in the number of *M.hominis* per cell. This may reflect a possible different capability of bacteria to infect *T.vaginalis* or a different susceptibility of *T.vaginalis* isolates to *M.hominis* infection. *T.vaginalis* is able to pass mycoplasmal infection *in vitro* not only to mycoplasma-free protozoan isolates, but also to epithelial cells derived from the human uterine cervix (43). This finding suggests a potential role for *T.vaginalis* as a carrier in transmitting the bacterial infection *in vivo*. The demonstration of ability of *M.hominis* to enter, survive and replicate within *T.vaginalis* cells (44), furtherly support this hypothesis.

In the past years the issue whether mycoplasmas localize intra- or extracellularly has long been debated (45). Interesting investigations showed that several pathogenic mycoplasmas species are intracellularly located (46, 47, 48). Differential immunostaining of intra- and extracellular mycoplasmas clearly shows the ability of *M.hominis* to enter *T.vaginalis* cells (Figure 1). Intracellular location may

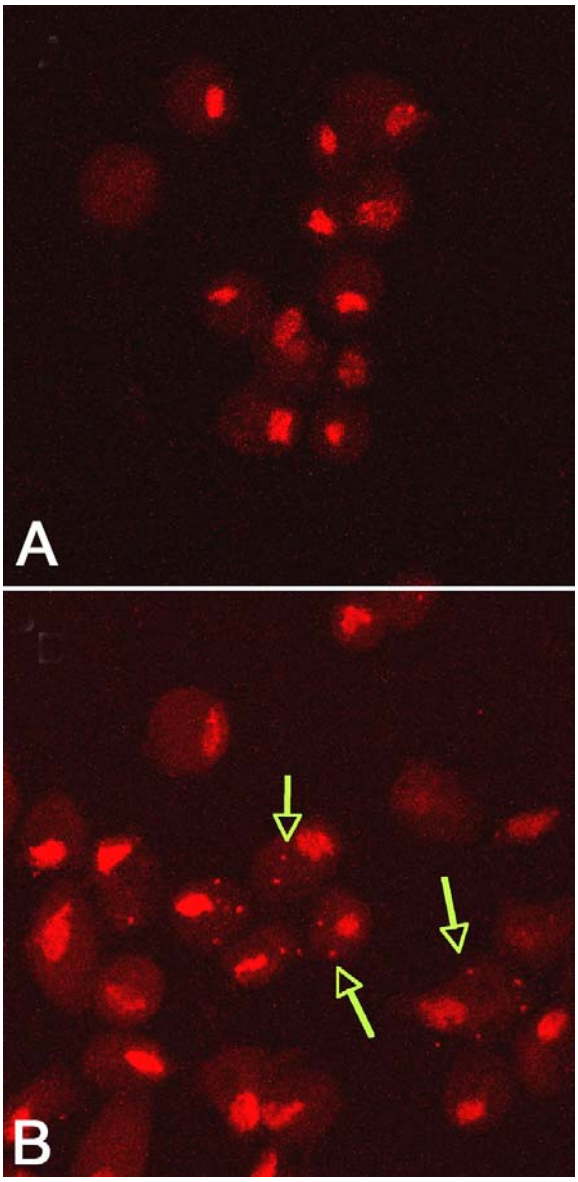

Figure 2. Detection of 5-BrdU incorporation by *M.hominis* located within *T.vaginalis* cells. A. mycoplasma-free *T.vaginalis* isolate and B. *M.hominis* -infected *T.vaginalis* after 48 hours of incubation with 5-BrdU and gentamicin. Incorporation has been highlighted using anti-5-BrdU antibodies. DNA biosynthesis is detectable in both *T.vaginalis* strains nuclei and in the cytoplasm of *Mycoplasma*-infected strain SS14, as indicated by arrows. Intracellular persistence of *M.hominis* over extended periods was demostrated with long-term gentamicin protection assay. Reproduced with permission of American Society for Microbiology (ASM) (44).

protect these bacteria from host immune response and antibiotic treatment and may also partially explain the difficulty in eradicating mycoplasma infections.

At present we are not still able to assess if *M.hominis* actively invades *T.vaginalis* or is phagocytized by the protist. Beside its ability to locate and survive within *T.vaginalis* cells *M.hominis* is also able to replicate in its intracellular environment. Gentamicin protection experiments coupled with 5-BrdU incorporation assay showed that mycoplasmas persist in their intracellular location and actively multiply (Figure 2). Figure 1 and figure 2 show that *M.hominis* cells seem to be concentrated in few hotspots and not dispersed throughout the *T.vaginalis* cell. In particular, figure 2 shows that mycoplasmas are preferentially located at the posterior end of trichomonad cells, where vacuoles can be detected. This suggests a possible localization of *M.hominis* in *T.vaginalis* endosomes/vacuoles, similarly to HIV particles ingested by endocytosis (49). This is precisely where mycoplasma-like structures have been observed in the first electron microscopy studies on *T.vaginalis* (28, 29). *M.hominis* replication within *T.vaginalis* cells represents a potential defence mechanisms during infection *in vivo*. Entry into protozoan cells may provide a protected niche for *M.hominis* survival. This may explain at least in part the ability of *M.hominis* to persist in the harsh environmental conditions of the vaginal tract (50). Furthermore, *T.vaginalis* may be viewed indeed as a "Trojan horse" in transmitting *M.hominis* infection *in vivo*.

Are Mollicutes other than *M.hominis* capable to establish a symbiotic relationship with protozoan species? *M.hominis* appears to be unique from this point of view, despite the observation of mycoplasma-like particles on the surface of *Giardia spp*.(51). This is a mere morphological description and putative mycoplasma microorganisms have never been isolated from *Giardia*, nor has this association been characterized. The biological association between *T.vaginalis* and *M.hominis* can not be only explained by the mere copresence in the vaginal tract. Mycoplasmas show a strict host and tissue specificity due to their limited genomes. Unlike the sexually transmitted mycoplasmas *M.genitalium* and *U.urealyticum*, *M.hominis* may have distinctive features which allow the establishment of a specific association with *T.vaginalis*.

4. CONCLUSIONS AND PERSPECTIVE

Mycoplasmas have been described as ubiquitous microorganisms infecting mammals, birds, reptiles, fish, arthropods, plants (52). *M.hominis* may have found a new niche within its natural habitat represented by the human urogenital tract: the pathogenic protist *T.vaginalis*. This phenomenon is another example of the versatility of Mollicutes. In fact, these small and simple but somehow complex microrganisms show a high degree of adaptation to the most diverse and adverse environments.

So far, the symbiotic relationship between *M.hominis* and *T.vaginalis* has been characterized only in part. Further studies will be needed to answer several questions. A major subject for future research should be the study of physiological and nutritional interchanges occurring between *M.hominis* and *T.vaginalis*. In fact, such interactions might confer to one or both the symbonts some advantage that could partly explain the establishment of the symbiosis. *T.vaginalis*, like *M.hominis*, is an obligate

parasite in that it lacks the ability to synthesize many macromolecules *de novo*, particularly purines, pyrimidines and many lipids. These nutrients are acquired by feeding on vaginal secretions and bacterial cells from the vaginal flora (53). Evidently, some biological features of one or both microorganisms allow *M.hominis* to change its condition from "food" to symbiont for *T.vaginalis*. In order to reach a deeper understanding of these issues, a characterization of *M.hominis* internalization mechanisms is needed. It should be assessed whether entry within *T.vaginalis* cells is a passive or active phenomenon. At the same time, determining the subcellular localization of mycoplasmal cells may help to better understand all the complex interactions occurring between *T.vaginalis* and *M.hominis*. Assessing whether mycoplasmas are free in the cytoplasms or reside in *T.vaginalis* vacuoles is pivotal. Compartimentalization within membrane-bound vacuoles might have led to the development of some defensive mechanism allowing *M.hominis* to avoid *T.vaginalis* lytic effectors. This may be viewed as another example of a protist species acting as "training ground" for intracellular bacteria.

An unvaluable help in deciphering the complex interaction between *T.vaginalis* and *M.hominis* should come from the recent complete sequencing of *T.vaginalis* genome (54). The ever growing informations coming from the sequencing project (http://www.tigr.org/tdb/e2k1/tvg/) will provide useful tools to study *T.vaginalis/M.hominis* interactions.

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